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Molecular mechanism of mutagenesis induced by olaquindox using a shuttle vector pSP189/mammalian cell system

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Abstract

Olaquindox, a quinoxaline 1,4-dioxide derivative from quindoxin, is widely used as an animal growth promoter in China. We tested olaquindox as a mutagen in a SV40-based shuttle vector pSP189 and African green kidney cell (Vero E6 cell line) system to define the safety of olaquindox as a food-additive for animals. When applied at 6.6 μg/ml, olaquindox caused 12 times higher mutation frequency in comparison to untreated controls. More than 70% of base substitutions happened at G:C base pairs featuring G:C to T:A or G:C to A:T conversions. Frequency of point mutations for in vitro modified plasmids was also dramatically increased from the spontaneous background level. Olaquindox-induced mutations did not occur randomly along the *supF* shuttle vector, but instead, had a hot spot at base pair #155 which accounts for 37% of total mutations. Olaquindox-induced mutations also showed sequence-specificity in which most point mutations occurred at site N in a 5′-NNTTNN-3′ sequence while most tandem bases deletion and rearrangement were seen at the 5′-ANGGCCNAAA-3′ sequence. We conclude that olaquindox induces DNA mutation, therefore, should not be used as an additive to promote animal growth.

Keywords: Shuttle vector plasmid; Olaquindox; Mutation; Vero cell

1. Introduction

Olaquindox is a synthetic quinoxaline 1,4-dioxide derivative with strong anti-microbial activities (Fig. 1). Because of its functional property, this substance has been broadly used in China to promote animal growth. While the beneficial effects are obvious, the usage of olaquindox in animal production raises serious concerns since previous studies have shown that quindoxin and its metabolites are potential mutagens [1]. It has been found that the mutagenicity of quinoxaline-di-N-oxides was dependent on the presence of their N-oxide groups [2–4]. When quindoxin, carbadox and olaquindox were subjected to the Chinese hamster V79/SCE test, all three chemicals caused dose-dependent increases in sister-chromatid exchange (SCE) frequency [5,6]. In a short-term test, feeding 4-week-old hybrid piglets with 0, 25, 50, 100 and 200 ppm olaquindox for 6 weeks had found dry faeces, drinking of urine from the floor of pens, a decrease in abdominal volume. Significant rises in serum albumin values and marked rises in serum urea values occurred in the 200 ppm group from week 4 and in the 100 ppm group from week 5. Gross and microscopic pathology were not conducted [7]. In a dominant lethal assay, olaquindox administrated orally at 200 and

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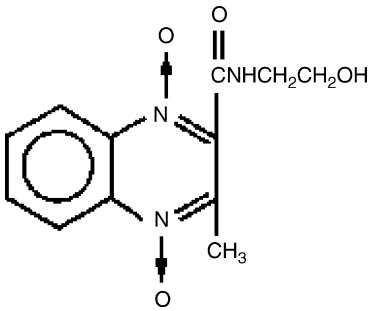

Fig. 1. Structure of Olaquindox.

500 mg/kg produced positive result [8]. The Commission of the European Community forbad the usage of olaquindox and carbadox as animal growth promoters in 1999, citing that no threshold can be established for the safe usage of these genotoxic additives, and that a small quantity of residues could provoke a tumour-inducing mutation [9].

In recent years, the rapidly growing Chinese economy accompanies a higher demand in animal products. As a result, a large array of growth promoters, including olaquindox, have been used to enhance animal production and to speed-up turn-over time in the food animal industry. We conducted this study to demonstrate the mutagenicity and mutation spectra of olaquindox in mammalian cells, aimed to find scientific evidence to safeguard its application in animal production. We used shuttle vector plasmids carrying mutational target genes to test olaquindox-induced mutation, a system that has been well established in mutation research [10–12], and is capable of determining the mutational specificities in mammalian cells induced by various mutagens [13–17]. For indirectly acting chemical mutagens, a chemically activated form of the mutagen is used to first treat the plasmid in vitro followed by transfection of the treated plasmid into culture cells to allow plasmids replication and conversion of premutational lesions into mutations. The nature of mutation is detected by DNA sequence analysis. We found from this study that olaquindox causes significant increase in mutation frequency and caution its continuous usage as a food additive for food animals.

2. Materials and methods

2.1. Chemicals

Olaquindox ($C_{12}H_{13}N_3O_4$, FW 263.25, CAS no. 6960; 99%) was obtained from China Institute of Veterinary Drug Control, and was dissolved in dimethylsulfoxide (DMSO). Ampicillin, 5-bromo-4-chloro-3-indolyl-β-D-galactoside (X-gal), isopropyl-β-D-thiogalactoside (IPTG) and the QIAprespin Plasmid Kit were obtained from Promage (Madison, WI, USA). Restriction endonuclease *DpnI* was purchased from Invitrogen (Carlsbad, CA, USA).

2.2. Cell culture

A SV-40-transformed Vero cell line (African green monkey kidney cell line) was obtained from Academy of Medical Sciences of Zhejiang Province (Hangzhou, China). Cells were cultured at 37 °C with 5% CO_2 in Dulbecco's modified Eagle's medium (Invitrogen, Carlsbad, CA) supplemented with 10% fetal bovine serum (Hyclone, Logan, UT), 100 U/ml penicillin and 100 U/ml streptomycin.

2.3. Shuttle vector plasmid and bacterial strains

The pSP189 shuttle vector, which carries a Lac Z amber mutation, was used for analysis of mutations in Vero cells. The indicator *E. coli* MBM7070 was used for detection of the mutated pSP189. Both the plasmid and the indicator were gifts from Dr. M.M. Seidman (NIH9900, USA).

2.4. Treatment of pSP189 with olaquindox, transfection and retrieval

A purified stock of pSP189 plasmid was prepared by using the Plasmid Purification Kit from Promage (Madison, WI, USA). The plasmids were treated with 6.6 μg/ml [18] of olaquindox and 0.1% of DMSO in 0.5 ml of pH 8.0 TE buffer, and were kept for 6 h at 37 °C in the dark [19]. The plasmids were precipitated by ethanol, filtered through SUPREC TM-02 (Takara, Kyoto, Japan) to remove excess olaquindox, and re-dissolved in 30 μl of TE buffer (pH 8.0).

Cultured Vero cells were trypsinized, washed and resuspended in Dulbecco's phosphate-buffered saline (PBS) solution (pH 7.5). Two groups of 5×10^5 Vero cells were each transfected with either olaquindox-treated or DMSO-treated pSP189 (8 μg) plasmid using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA). Cells were then plated in 25 ml culture dishes and incubated at 37 °C for 4 h. After Lipofectamine–DNA co-precipitates were removed, cells were washed and cultured for 48 h in fresh medium. Plasmid DNA was extracted with the Promage Plasmid Purification Kit, and was digested with DpnI (Promage, 20 U) to eliminate the non-replicated plasmids which retain the bacterial methylation pattern.

2.5. Characterization of induced mutations

The progeny plasmids were transfected into *E. coli* MBM7070 by using calcium chloride. *E. coli* bacteria were plated on Luria–Bertini (LB) agar plates containing ampicillin (100 μg/ml), X-gal (50 mg/ml) and IPTG (200 mg/ml). A por-